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LPS-mediated septic shock is augmented in ceramide synthase 2 null mice due to elevated activity of TNFα-converting enzyme

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ABSTRACT

Tumor necrosis factor α (TNFα) is an inflammatory cytokine that plays an intimate role in septic shock. Injection of high levels of lipopolysaccharide induces septic shock and death in mice within 30 h, whereas ceramide synthase 2 (CerS2) null mice, defective in the synthesis of very-long acyl chain ceramides, die within ~10 h. The augmented rate of death of CerS2 null mice is due to elevated levels of TNFα secretion as a result of enhanced activity of TNFα-converting enzyme (TACE). We discuss the relationship between the sphingolipid acyl chain length and TACE activity and the relevance of this data to septic shock.

1. Introduction

Due to its potent polytrophic effects on survival, apoptosis, and necroptosis, tumor necrosis factor α (TNFα) secretion is tightly regulated [1]. Mis-regulation of TNFα secretion has been linked to various diseases and pathologies including sepsis, rheumatoid arthritis and cancer [2]. In mice, TNFα mRNA is translated into a 26kDa precursor protein (pro-TNFα) which is transported to the plasma membrane where it resides as a type II membrane protein [3]. The pro-TNFα ecto-domain is cleaved by TNFα-converting enzyme (TACE) prior to its release as a biologically-active 17kDa soluble form [4,5].

We now examine the effect of altering the ceramide/sphingolipid (SL) acyl chain length on TNFα secretion and TACE activity in a model of sepsis. This study was performed using ceramide synthase 2 (CerS) null mice, which are unable to generate very-long chain (C22–C24) ceramides [6,7]. CerS2 null mice display increased rates of hepatocyte death and proliferation, resulting in formation of multiple hepatic nodules, and in some cases, hepatocellular carcinoma [7], along with chronic oxidative stress [8] and hepatic insulin resistance [9], which are all likely due to changes in membrane biophysical properties [10]. Recently, we demonstrated that altering the SL acyl chain composition in CerS2 null mice prevents lipopolysaccharide (LPS)/galactosamine (GLN)-mediated hepatic failure by disrupting TNFR1 internalization [11]. We now show that in an LPS-induced model of sepsis, CerS2 null mice secrete high levels of TNFα due to enhanced TACE activity, which results in rapid death of the animals upon LPS-mediated septic shock.

2. Materials and methods

2.1. Reagents

A rabbit polyclonal anti-TACE antibody was from Abcam (Cambridge, UK). LPS was from Sigma–Aldrich (St. Louis, MO, USA). TACE substrates were from R&D (Minneapolis, MN, USA). A TNFα ELISA kit was from Biolegend (San Diego, CA). Macrophage colony stimulating factor was from Peprotech (Rocky Hill, NJ).

2.2. Mice

CerS2 null mice were generated and maintained as described [11]. Mice were treated in accordance with the Animal Care

Abbreviations: BMDM, bone marrow derived macrophage; CerS2, ceramide synthase 2; LPS, lipopolysaccharide; SL, sphingolipid; TIMP3, tissue inhibitor of metalloproteinases 3; TNFα, tumor necrosis factor α; TACE, tumor necrosis factor α converting enzyme

Author contributions: MA performed most of the experiments and AS contributed some data. YPJ helped supervise the project along with AHF, who also wrote the paper.

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Guidelines of the Weizmann Institute of Science Animal Care Committee, and the National Institutes of Health's Guidelines for Animal Care. Mice were injected intraperitoneally with 35 mg/kg LPS. Liver sections were collected for histological examination or were flash frozen. Blood was collected via the orbital sinus for analysis of TNFα secretion. Serum TNFα levels were measured using an anti-mouse enzyme-linked immunosorbent assay kit (BioLegend, San Diego, CA).

2.3. Hepatocyte isolation

Hepatocytes were isolated from mice after hepatic portal vein perfusion using warm Ca^{++}- and Mg^{++}-free Hank's balanced salt solution (Sigma–Aldrich) containing 5.5 mM KCl, 5.5 mM glucose, 25 mM $NaHCO_3$, 0.7 mM EDTA for 3 min, to which liver digest media (GIBCO/BRL Life Technologies, Carlsbad, CA, U.S.A) was added for a further 8 min. After perfusion, the liver was quickly excised and the gall bladder removed. Hepatocytes were separated from connective tissue using sterile tweezers and then passed through a cell strainer (BD Falcon Labware, Franklin Lakes, NJ USA) and centrifuged at $50 \times g_{av}$ (4 °C, 5 min). Hepatocytes were suspended in DMEM containing 10% fetal bovine serum, 2 mM sodium pyruvate, 2% penicillin/streptomycin and 1 μM dexamethasone.

2.4. Isolation of bone marrow derived macrophages

2–3 month-old mice were euthanized and the tibia and fibula collected for the extraction of bone marrow. Bone marrow cells were cultured in RPMI medium (Gibco, Invitrogen, USA) supplemented with 15% fetal bovine serum, 1% sodium pyruvate, 1% penicillin/streptomycin and 10 ng/ml macrophage colony stimulating factor, and cultured for 7 days [12]. Bone marrow-derived macrophage (BMDMs) were serum-starved for 5–8 h.

2.5. Western blotting

Tissue lysates were prepared in radioimmunoprecipitation assay buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 50 mM NaF and 2 mM Na_3VO_4. Protein was measured using the BCA Protein Assay Kit (Pierce Chemical Co., Carlsbad, CA, USA). Fifty microgram of protein was loaded and separated on 8–15% SDS–PAGE and transferred to a nitrocellulose or PVDF membrane. The membrane was blocked using 5% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween-20 (PBST) for 1 h at room temperature. The primary antibody was diluted in PBST containing 1% bovine serum albumin and incubated with the membrane at 4 °C overnight. After 3 washes with PBST, membranes were incubated with the secondary antibody in PBST containing 1% BSA at room temperature for 1 h.

2.6. Quantitative real-time PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to manufacturer's instructions. cDNA synthesis was performed using a Reverse-iT first strand synthesis kit (Thermo Scientific, Grand Island, NY, USA) using random hexamers. qPCR was performed using a SYBR Green PCR Master Mix (Finnzyme, Life Technologies, Vantaa, Finland) with cDNA (equivalent to 5 ng of total RNA) and an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Life Technologies).

The following primers were used:

IκBα:	Forward: TTGGTCAGGTGAAGGGAGAC; Reverse: ACAGCCAAGTGGAGTGGAGT.
A20:	Forward: GGTGATGGAAACTGCCTCAT; Reverse: CTTCCTCAGGACCAGGTCAG.
cIAP2:	Forward: CGAGGAGGAGGAGTCAGATG; Reverse: GGAGGCAATACAGCATTGGT.
TNFα:	Forward: CTTGTGGCAGGGGCCACCAC; Reverse: CCATGCCGTTGGCCAGGAG.

2.7. Confocal microscopy

Confocal microscopy was performed using an Olympus IX 81 Fluo-View 1000 microscope and a UPLSAPO 60× objective.

2.8. Statistical analysis

Values are expressed as means ± S.E.M. Statistical significance was calculated using the Student's *t* test.

3. Results and discussion

Injection of high doses of LPS (35 mg/kg) lead to the death of WT mice within 30 h whereas CerS2 null mice died within 10 h, with a mean time of death of 5.4 ± 4.3 h compared to 17.7 ± 4.8 h in WT mice (Fig 1). LPS treatment causes secretion of TNFα which leads to secretion of proinflammatory cytokines (the 'cytokine storm') resulting in sepsis [13]. TNFα levels in serum from CerS2 null mice were ~6-fold higher than in WT mice 1.5 and 3 h after LPS treatment (Fig. 2).

To determine whether increased levels of serum TNFα might result from increased secretion from macrophages, bone marrow cells were treated with macrophage colony stimulating factor to differentiate them into bone marrow-derived macrophages (BMDMs) [12], followed by treatment with 200 ng/ml LPS. BMDMs from CerS2 null mice secreted higher amounts of TNFα than BMDMs from WT mice (Fig. 3A); however, there was no change in levels of TNFα mRNA (Fig. 3B) or protein (not shown), no changes in TLR4 activation as estimated by ERK1/2 phosphorylation (not shown), and no changes in levels of expression of genes

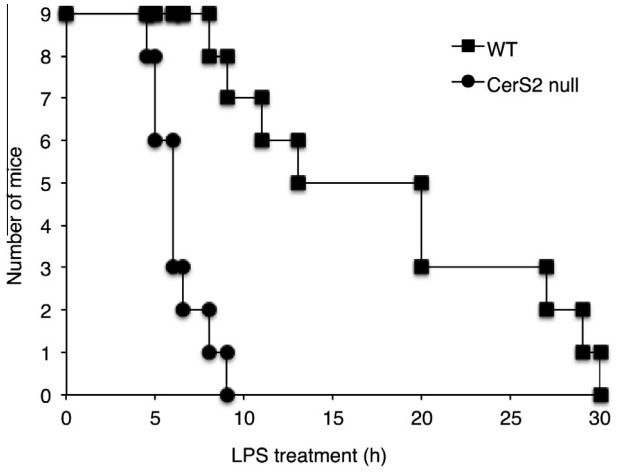

Fig. 1. Sensitivity of Cers2 null mice to LPS-mediated septic shock. 2–3 month-old WT and CerS2 null mice were injected intraperitoneally with a single dose of LPS (35 mg/kg) and the survival of the mice was determined. $n = 9$.

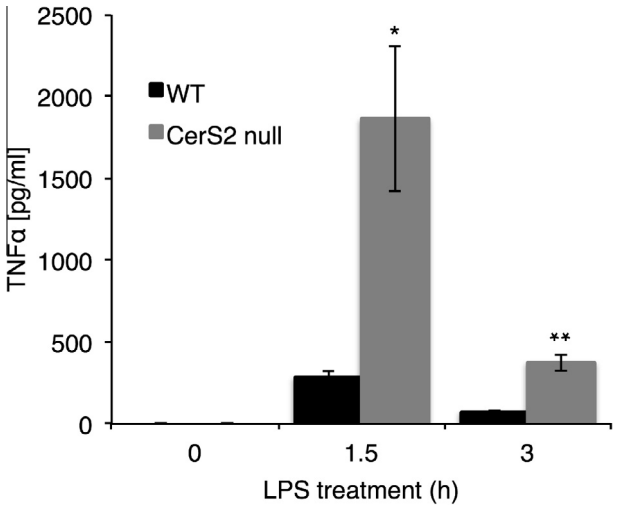

Fig. 2. Serum TNFα levels after LPS treatment. Serum TNFα levels were measured at 1.5 and 3 h after LPS treatment (as in Fig 1). $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.005$.

downstream to NFκB (IκBα, Fig. 3B; A20 and cIAP2, not shown). These results are consistent with altered TNFα levels being a result of altered TNFα processing. Indeed, TACE activity was significantly elevated in BMDMs (Fig. 4A), without any change in intracellular distribution of TACE (Fig. 4B). TACE activity was also elevated in CerS2 null liver (6.3 ± 1.4 μmol/mg/min in CerS2 null liver compared to 3.4 ± 0.3 μmol/mg/min in WT liver; $n = 4$) and in hepatocytes isolated from CerS2 null (1.86 ± 0.15 μmol/mg/min) compared to WT mice (1.14 ± 0.27 μmol/mg/min; $n = 4$), which corresponded with increased levels of TNFα secretion both before and after treatment with 500 ng/ml LPS (Fig. 5A). However, levels of TACE protein were unaltered (Fig. 5B), confirming the notion that TACE activity is modulated via a post-translational mechanism, which is presumably related to the altered SL acyl chain length.

Previously we demonstrated that CerS2 null mice were protected against LPS/GLN-mediated fulminant hepatic failure due to disrupted TNFR1 internalization, which resulted in inhibition of caspase 8 activation. However, TNFR1-mediated NFκB signaling is not altered in CerS2 null mice [11], which is consistent with activation of NFκB inflammatory pathways [14] due to excessive TNFα secretion. Although we did not determine the precise mechanistic relationship between the SL acyl chain length and TACE activity, a number of possibilities could explain our results. TACE is negatively regulated by tissue inhibitor of metalloproteinase 3 (TIMP3), a 25kDa secreted protein that interacts strongly with the extra-cellular matrix [15,16]. TIMP3$^{-/-}$ mice exhibit high levels of TACE activity and as a result secrete elevated levels of TNFα, which results in liver necrosis and liver failure [17,18]; indeed, CerS2 null mice show a 45 ± 14% ($n = 6$) reduction in levels of TIMP3 in the liver. Previous studies have shown that altering the SL composition directly affects TACE activity; thus, TACE is hyper-activated in acid sphingomyelinase −/− cells, and exogenously-added acid sphingomyelinase inhibits TACE activity, as does exogenously-added short acyl chain ceramide [19]. Moreover, ceramide 1-phosphate binds TACE directly and down-regulates its activity [20], although this is unlikely to be the mechanism involved in TACE regulation in the current study as ceramide 1-phosphate levels are essentially unchanged (data not shown). Thus, there is significant evidence that altering the SL composition, and in particular ceramide levels, can modulate TACE activity; our study suggests that altering the SL acyl chain length increases TACE activity although whether this is specific to the changes in the SL composition observed in CerS2 null mouse, or might occur in other CerS null mice, is currently unknown. Importantly, TACE is regulated by membrane components, with lipid raft depletion resulting in elevated TACE activity [21], and lipid rafts are disrupted in CerS2 null mice (as ascertained by changes in cholera toxin binding [9]); it is possible that TIMP3 is unable to interact with TACE in lipid rafts. Irrespective of the precise mechanism by which TACE activity is elevated in CerS2 null mice, our study indicates a direct link between SL composition and TACE activation, which may impact upon processes in which TACE is involved such as sepsis, inflammation and cell tumorogenesis.

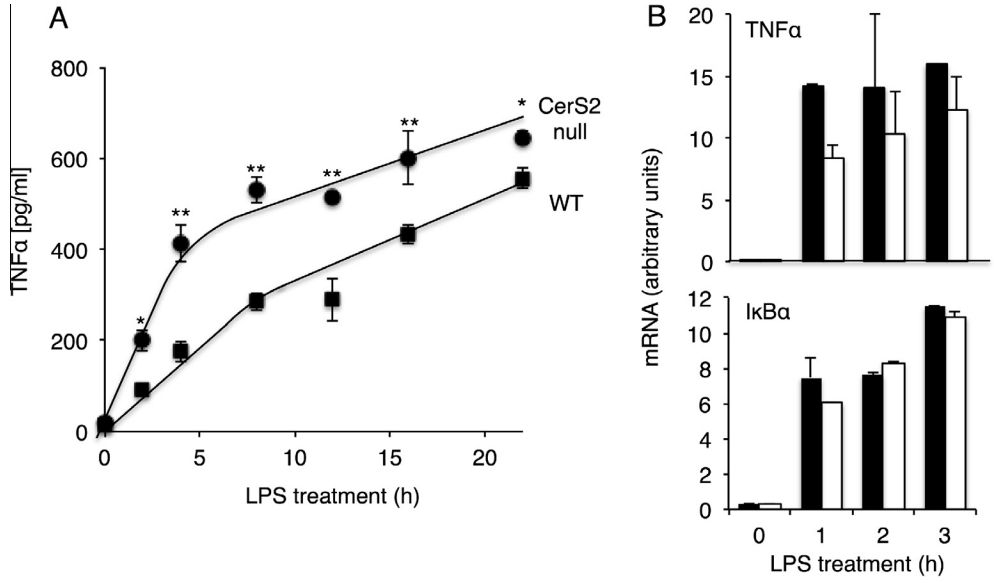

Fig. 3. TNFα secretion from BMDMs. BMDMs were incubated with 200 ng/ml LPS. (A) TNFα levels in the medium. $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.005$. (B) TNFα and IκBα mRNA levels in BMDMs. $n = 4$, $^{*}P < 0.05$.

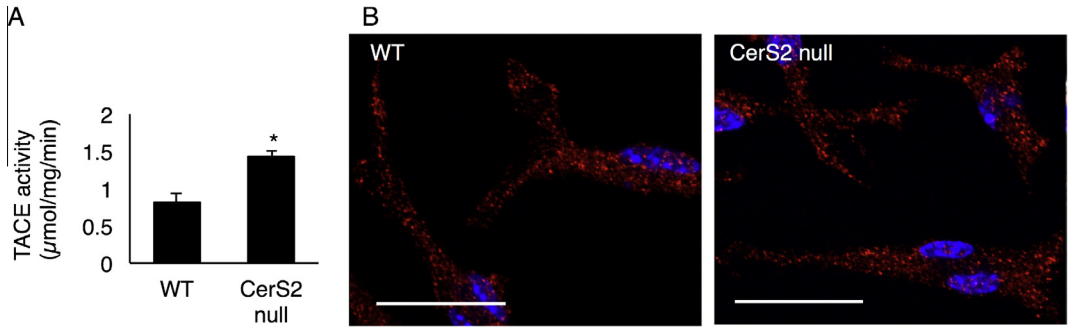

Fig. 4. TACE activity and intracellular localization. (A) TACE activity was measured in BMDMs from WT and CerS2 null mice. $n = 4$, $^{*}P < 0.05$. (B) TACE localization (red) detected by confocal microscopy; blue (DAPI) indicates nuclei. Scale bar, 20 μm.

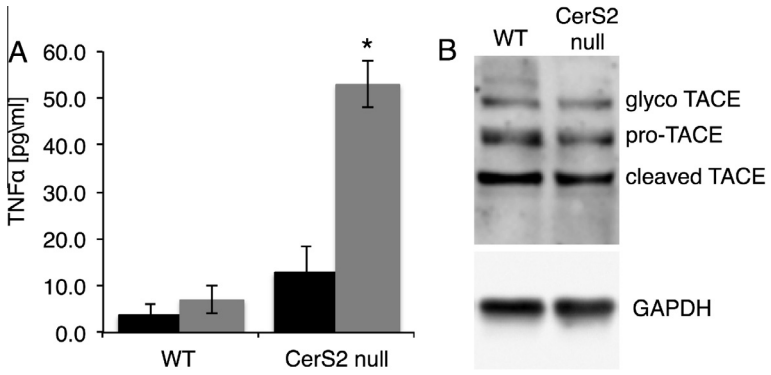

Fig. 5. (A) TNFα secretion and levels in hepatocytes and liver. (A) Black columns show basal TNFα levels and gray columns show TNFα levels 60 min after LPS (500 ng/ml) treatment. $n = 3$, $^{*}P < 0.005$. (B) Western blot of TACE in liver homogenates. The different forms of TACE are identified. The experiment was repeated 5 times and gave similar result.

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